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The t-complex polypeptide 1 complex is a chaperonin for tubulin and actin *in vivo*

(protein folding/pulse–chase studies/cytosolic chaperone)

HIMAN STERNLICHT*†, GEORGE W. FARR*, MONA L. STERNLICHT*, JANE K. DRISCOLL*, KEITH WILLISON‡, AND MICHAEL B. YAFFE*§

*Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106; ‡Institute of Cancer Research, Chester Beatty Laboratories, London SW3 6JB, United Kingdom; and §Department of Surgery, University Hospitals of Cleveland, Cleveland, OH 44106

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ABSTRACT **A role in folding newly translated cytoskeletal proteins in the cytosol of eukaryotes has been proposed for t-complex polypeptide 1 (TCP1). In this study, we investigated tubulin and actin biogenesis in Chinese hamster ovary (CHO) cells. When extracts of pulse-labeled cells were analyzed by anion-exchange and size-exclusion chromatography, newly synthesized α-tubulin, β-tubulin, and actin were observed to enter a large molecular mass complex (≈900 kDa). These proteins were released from this complex capable, in the case of tubulin, of forming heterodimers. The large molecular mass complexes coeluted with TCP1 and could be immunoprecipitated by using an anti-TCP1 antibody. These findings demonstrate that there is a cytosolic pathway for folding tubulin and actin *in vivo* that involves the TCP1 complex.**

The eukaryotic cytoskeleton composed of intermediate filaments, microtubules, and microfilaments is essential for a variety of cellular processes including mitosis, motility, and maintenance of cell shape (1). Despite its critical importance for cellular function, little is known regarding the biogenesis of its constituent proteins (2–4). For many polypeptides, molecular chaperones and heat shock proteins are essential for proper folding and assembly into oligomeric complexes (5, 6). Previous somatic cell genetic studies implicated the 70-kDa heat shock protein (hsp70) and the mitochondrial hsp60 chaperonin (mt-hsp60) in tubulin folding and microtubule formation, but this model requires tubulin transit into and out of the mitochondria (7).

TCP1 complex is an oligomeric particle (≈900 kDa) found in the eukaryotic cytosol consisting of t-complex polypeptide 1 (TCP1) and four or five related polypeptides of similar size (55–60 kDa) (8, 9) and has recently been proposed to be the cytosolic equivalent to GroEL and mt-hsp60 (10, 11). A role for TCP1 complex as a chaperonin in the eukaryotic cytosol is suggested by *in vitro* studies demonstrating function of the complex in (*i*) refolding urea-denatured tubulin and actin (12, 13) and (*ii*) folding newly translated α- and β-tubulin in rabbit reticulocyte lysates (14). Our study indicates that in CHO cells newly synthesized α- and β-tubulin and actin enter a 900-kDa complex that contains TCP1 and that tubulin is released from this complex competent to form heterodimers. Taken together with *in vitro* studies of tubulin and actin folding (12–14) and the observation that mitotic spindle assembly is impaired in TCP1 mutants of *Saccharomyces cerevisiae* (15), these findings strongly argue that the TCP1 complex is a cytosolic chaperonin, one of whose functions *in vivo* is folding tubulin and actin.

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MATERIALS AND METHODS

Labeling and Extraction of CHO Cells. CHO cells (5×10^5 to 1×10^6 cells per dish) were grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum. Cells were labeled for the indicated times in methionine-deficient medium supplemented with [^{35}S]methionine (200–500 μCi; 1 Ci = 37 GBq). Radiolabel incorporation was terminated by flooding the plates with complete medium supplemented to 10 mM unlabeled methionine. The labeled cells were extracted *in situ* on ice (10 min) using 200 μl of extraction buffer (FB buffer; 100 mM KCl/10 mM Pipes, pH 6.8/300 mM sucrose/2 mM $MgCl_2$/1 mM EGTA) (4) containing 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin. A modified extraction buffer at pH 7.2 with Hepes (15 mM) replacing Pipes (10 mM) was used for immunoprecipitation studies (see below).

Immunoblots. CHO cells (≈$0.8–2 \times 10^8$ total cells) were treated with trypsin and extracted in 500 μl of FB buffer (10 min on ice). Clarified extracts were chromatographed on a Superose 6 HPLC column following Yaffe *et al.* (14). Fractions were dialyzed against ammonium bicarbonate (1 mM; pH 6.8) and concentrated for SDS/PAGE. Immunoblots were probed with anti-hsp60 antibody P_1-3 (17), stripped, and reblotted with anti-TCP1 antibody 91A (8). Immunoreactive proteins were detected by the enhanced chemiluminescence (ECL) procedure (Amersham).

Immunoprecipitations with Anti-β-Tubulin Antibody KMX-1. Fractions I–III from anion-exchange chromatography (see Fig. 1) were immunoprecipitated with monoclonal antibody KMX-1 following Harlow and Lane (18) (see Fig. 1*C*). Pansorbin-precipitated immune complexes were washed under mild conditions [phosphate-buffered saline (PBS) containing 0.05% Nonidet P-40 (NP-40)] prior to extraction in SDS/PAGE sample buffer. Later studies established that tubulin–TCP1 complexes are stable at 4°C and subsequent immunoprecipitations done with the anti-TCP1 antibody 23C (see Fig. 4) used more stringent wash conditions.

Immunoprecipitations with Anti-TCP1 Antibody 23C. Control studies with reticulocyte lysate established that antibody 23C, which is directed against the C terminus of TCP1 (19, 20), is significantly more effective than antibody 91A for immunoprecipitation of β-tubulin–TCP1 complex (data not shown). Pulse-labeled or pulsed–chased CHO cell extracts (100 μl; ≈1×10^6 cells) were incubated for 3 hr at 4°C with 4 μl of antibody 23C (1.6 μg/μl) or PBS. Protein A-Sepharose (Sigma; no. P9424) (40 μl; 1:1 suspension) in NP-40 buffer (0.5% NP-40/150 mM NaCl/10 mM $MgCl_2$/15 mM Hepes, pH 7.5) was added and the samples were incubated for 1 hr at 4°C. The pellets were washed (five times) in 100 μl of NP-40 buffer and once in 100 μl of H_2O and then extracted with

Abbreviation: TCP1, t-complex polypeptide 1.

†To whom reprint requests should be addressed.

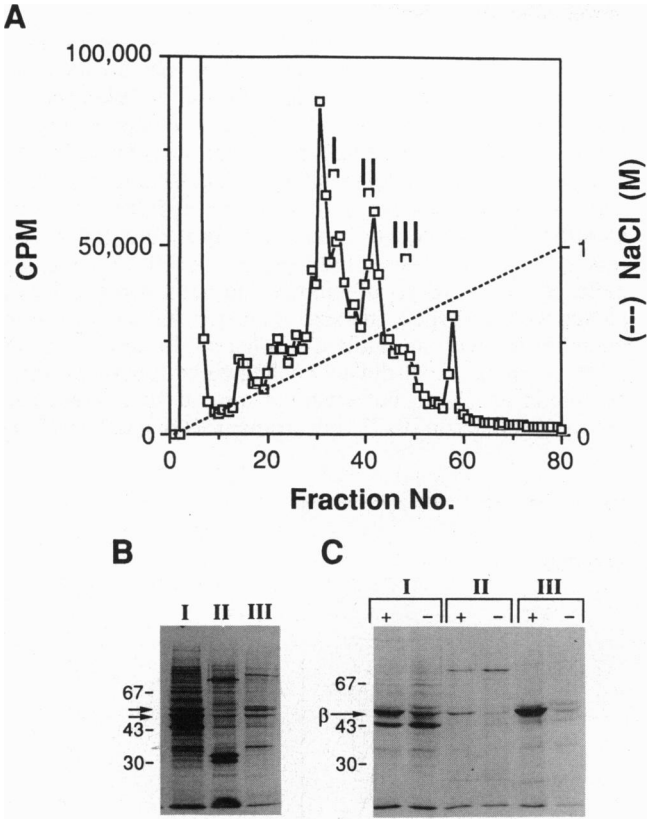

FIG. 1. Newly synthesized β-tubulin polypeptides in CHO cells exist in distinct molecular forms. CHO cell extracts were chromatographed on a Mono Q anion-exchange column, following Yaffe *et al.* (14). Fractions (0.5 ml) emerging at the positions of the β-polypeptide forms found in reticulocyte lysate (fractions I–III, respectively) (*A*) were collected, concentrated 10-fold, and either analyzed by SDS/PAGE (*B*) or immunoprecipitated with (+) or without (−) monoclonal antibody KMX-1 (Boehringer Mannheim) specific to β-tubulin (*C*). Arrows in *B* designate α- and β-tubulin positions based on bovine microtubule protein standards. The intense radiolabeled polypeptide at ≈43 kDa is assigned to actin (see *Discussion*).

either SDS/PAGE sample buffer (100°C; 10 min) or with isoelectric focusing buffer (minus ampholines) (70°C; 20 min).

RESULTS

Multiple α- and β-Tubulin Forms in CHO Cells. Previous *in vitro* studies showed that β-tubulin polypeptides synthesized in rabbit reticulocyte lysate are found in three molecular forms separable by anion-exchange chromatography (Mono Q): an early eluting form (β_I) corresponding to a complex between β-tubulin and a reticulocyte protein(s) and later eluting forms corresponding to the free β subunit (β_{II}) and the tubulin αβ heterodimer (β_{III}). β_I eluted from the Mono Q column as a mixture of two size classes: a 900-kDa form, which was the major form at early times of synthesis, and a 180-kDa form seen also at late times of synthesis (14). The 900-kDa form was shown to be a complex between newly translated β-tubulin and the TCP1 complex and to contain tubulin precursor to the free β subunit and tubulin heterodimer (14). A similar result was obtained for newly synthesized α-tubulin (14).

To determine whether these multiple forms of newly synthesized tubulin polypeptides also occur in intact cells, CHO cells were pulse-labeled with [^{35}S]methionine for 5 min and extracts were analyzed by anion-exchange chromatography (Fig. 1*A*). Fractions eluting at the β_I, β_{II}, and β_{III} positions (designated fractions I, II, and III in Fig. 1*A*) were collected, dialyzed, and concentrated for SDS/PAGE analysis. Fluorographs of fractions I and III revealed a large number of radiolabeled proteins, including several that ran coincident with α- and β-tubulin and actin standards (Fig. 1*B*). Immunoprecipitation studies with a monoclonal antibody specific to β-tubulin confirmed the presence of β-tubulin in fractions I, II, and III (Fig. 1*C*). However, in contrast to the reticulocyte lysate study (21), little β-tubulin was detected as the free subunit. Fractions taken at other positions in the anion-exchange salt gradient revealed no detectable tubulin.

Newly Synthesized Tubulin Subunits and Actin in CHO Cells Are Associated with a Large Molecular Mass Complex (≈900 kDa) Containing TCP1. When CHO cells were pulse-labeled for 3.5 or 12 min, the amount of radiolabeled β-tubulin in fraction I remained essentially constant, whereas a progressive increase of radiolabel was observed in fraction III (data not shown). This result suggested that fraction I contained the precursor of the other forms. This hypothesis was tested by pulse–chase experiments. CHO cells were labeled for 3.5 min and then chased in the presence of a 5000-fold molar excess of unlabeled methionine for 11 min. Analysis of these pulse–chase samples by two-dimensional SDS/PAGE confirmed the presence of α- and β-tubulin in fractions I and III, with little β-tubulin in fraction II (Fig. 2). However, in contrast to the immunoprecipitation study (Fig. 1*C*), the major β form at early times was in fraction I. At the end of the chase, radiolabeled α- and β-tubulin increased severalfold in fraction III, accompanied by a corresponding decrease of radiolabel in fraction I. A longer chase period increased the amount of α- and β-tubulin radiolabel detected in fraction III (data not shown). We interpret these results as evidence that fraction I contains a precursor to the tubulin heterodimer *in*

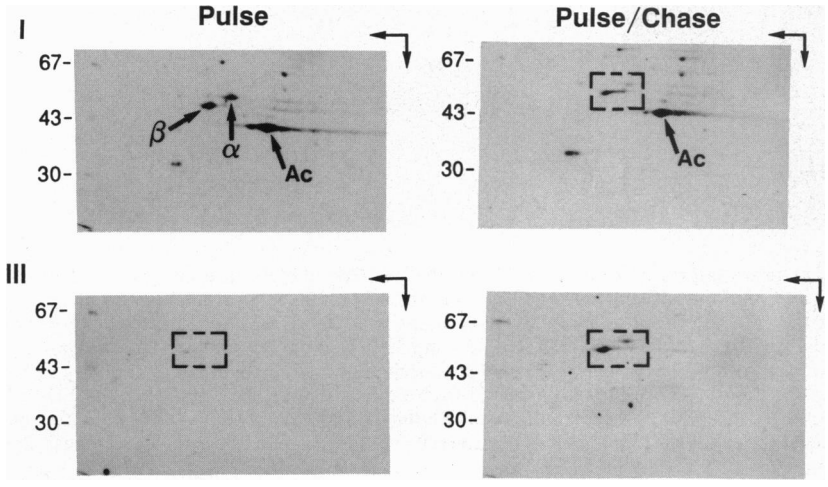

FIG. 2. Newly synthesized tubulin in fraction I chases into the tubulin dimer. CHO cells were pulse-labeled for 3.5 min and then chased for 11 min. The extracts were applied to the anion-exchange column. Fractions I–III were concentrated, supplemented with bovine microtubule protein and rabbit skeletal actin, and subjected to two-dimensional SDS/PAGE, stained with Coomassie blue, dried, and fluorographed. Positions of α- and β-tubulin and actin in the fluorographs were determined by overlay of the fluorographs on the corresponding Coomassie blue-stained gels. Boxed areas show the expected region for α- and β-tubulin.

vivo and estimated the half-life for conversion to be 5–10 min at 37°C in CHO cells. This estimated value is in good agreement with the 5- to 7-min half-life reported for the β_I form in reticulocyte lysate (14).

We previously demonstrated in rabbit reticulocyte lysate that newly translated α- and β-tubulin form a 900-kDa complex with TCP1 (14). To test whether the precursor α- and β-tubulin forms detected in CHO cells (Fig. 2) represented a similar complex with TCP1, pulse–chase extracts (5-min label, 12-min chase) were fractionated on a size-exclusion column (Fig. 3*A*). A peak of radioactivity at ≈900 kDa (fractions 62–67) was evident in the pulse-labeled but not in the pulsed–chased samples (Fig. 3*A*). Two-dimensional SDS/PAGE analysis demonstrated that tubulin and actin were the major radiolabeled species in this peak (Fig. 3*B Left*). These high molecular mass forms of tubulin and actin were reduced in the pulse–chase experiment (≥70% in the case of tubulin, >95% in the case of actin), indicating a precursor role in the biogenesis of tubulin and actin (Fig. 3*B Right*). Furthermore, immunoblot analysis revealed that this radiolabeled 900-kDa peak coeluted with the CHO cell TCP1 complex (Fig. 3*A Inset*). In addition, these high molecular mass forms in CHO cells were chromatographically indistinguishable from the α- and β-tubulin and actin complexes with TCP1 previously identified in reticulocyte lysate (unpublished data; see also refs. 12 and 14). To exclude the possibility that the high molecular mass forms of tubulin and actin represent complexes with mt-hsp60, the size-exclusion fractions were immunoblotted with an antibody to hsp60. In contrast to the TCP1 complex, hsp60 did not coelute with the 900-kDa forms of tubulin and actin but emerged instead as a broad peak centered at fraction 70–71 with an apparent mass of ≈475 kDa

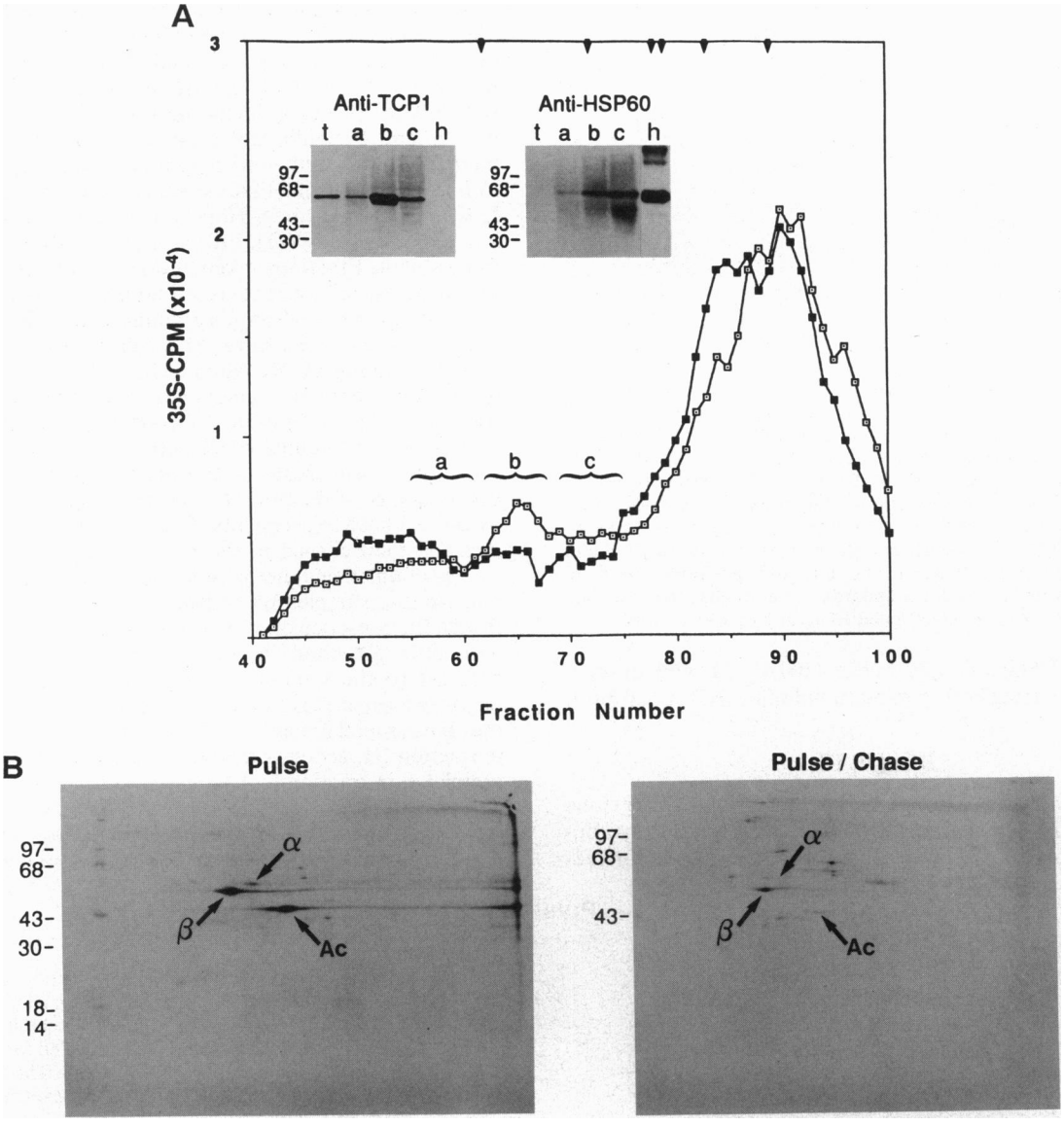

FIG. 3. Newly synthesized α- and β-tubulin and actin are associated with TCP1. (*A*) Normalized ^{35}S radiolabel profiles of extracts from pulsed (□) and pulsed–chased (■) CHO cells (*Materials and Methods*) (5-min pulse; 12-min chase; ≈2×10^6 cells) fractionated on a Superose 6 size-exclusion column. Fractions 62–67 (≈900 kDa) (b) were pooled and analyzed by two-dimensional SDS/PAGE fluorography (*B*). Arrowheads in *A* indicate positions of molecular mass standards. Left to right: 669, 440, 232, 158, 67, and 32 kDa. Void volume (≥3×10^4 kDa) at fraction 42. (*Inset*) Fractions 55–61 (a), 62–67 (b), and 69–75 (c) from ≈2×10^7 cells were subjected to immunoblot analyses with antibody 91A against TCP1 and antibody P_1-3 against mt-hsp60 (lanes a–c). Lane t, TCP1 complex isolated from rabbit reticulocyte lysate (≈0.4 μg) (14); lane h, recombinant human mt-hsp60 (PKK13B) (≈1 μg). P_1-3 and PKK13B were kind gifts of Rodhay Gupta (17). The 900-kDa value for the TCP1 complex is derived from a linear regression analysis of the semilogarithmic plot of mass versus elution position of the molecular mass standards (see ref. 14).

(unpublished studies; see also Fig. 3*A Inset*). This size agrees well with literature values (22) and presumably represents a homomeric complex of 7 rather than 14 identical subunits (22, 23). The difference in molecular mass between mt-hsp60 and the high molecular mass forms of tubulin and actin (Fig. 3*A*) argues against these forms being complexes with mt-hsp60. This conclusion is further supported by the absence of hsp60 in rabbit reticulocyte lysates (data not shown) and by the report that mt-hsp60 does not bind denatured tubulin (12).

Assignment of the high molecular mass tubulin and actin forms to complexes with TCP1 was confirmed by immunoprecipitations with anti-TCP1 antibody 23C (Fig. 4). CHO cells were labeled for 5 min and then either chased for 15 min *in vivo* (Fig. 4 *A* and *B*) or lysed and chased *in vitro* for 15 min in the presence or absence of EDTA (Fig. 4*B*). The binding of Mg^{2+} by EDTA inhibits ATP hydrolysis and arrests newly synthesized polypeptides on the cytosolic chaperonin (14). In the pulsed sample α- and β-tubulin and actin were the major radiolabeled products. Chased samples demonstrated a pronounced decrease of immunoprecipitable tubulin and actin, which was largely arrested with EDTA, confirming that these proteins are transiently associated with the TCP1 complex (Fig. 4*B*). Additional radiolabel proteins were also detected, primarily as a faint background with a number of weak bands (the latter representing <10% of the total radiolabel) (Fig. 4). However, with the exception of an ≈40-kDa protein, radiolabel levels in these proteins did not change during the chases (Fig. 4*B*). We therefore suspect that the majority of these proteins are not substrates of the TCP1 complex. In addition to TCP1, antibody 23C also cross-reacts with a 102-kDa subunit of the Golgi coatomer as well as with ≈63- and ≈115-kDa proteins (20). TCP1 and coatomer complexes are not detected in studies using short pulse times (Fig. 4; unpublished data). Whether the radiolabeled bands at ≈63 and ≈110 kDa correspond to cross-reacting polypeptides of 63 and 115 kDa is not known at this time.

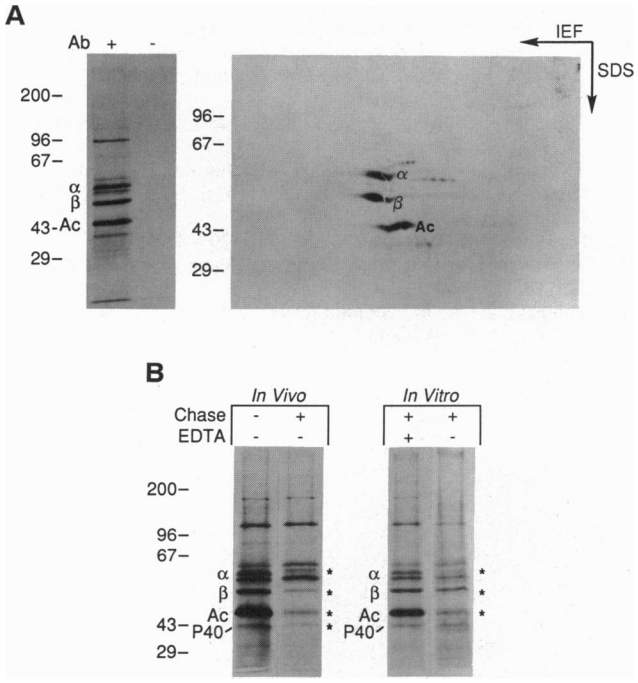

FIG. 4. Immunoprecipitations with anti-TCP1 antibody 23C confirm tubulin and actin (Ac) as the major substrates of the TCP1 complex. (*A*) CHO cells were pulse-labeled for 5 min, extracted, immunoprecipitated with (+) or without (−) antibody 23C, and analyzed by one- and two-dimensional SDS/PAGE. IEF, isoelectric focusing. (Carbamoylation, evident as multiple, closely spaced spots in the two-dimensional gel, occurred during urea extraction of the immunoprecipitates.) (*B*) Pulse–chase immunoprecipitates analyzed by one-dimensional SDS/PAGE. CHO cells were pulse-labeled for 5 min, chased for 15 min, extracted, and immunoprecipitated with antibody 23C (*in vivo* lanes) or pulse-labeled for 5 min, extracted, chased for 15 min in the presence or absence of 15 mM EDTA, and then immunoprecipitated (*in vitro* lanes). *, Proteins [α- and β-tubulin, actin, and p40 (40-kDa polypeptide)] whose radiolabel levels decreased during the chase.

DISCUSSION

Pulse–chase experiments revealed that in CHO cells newly synthesized α- and β-tubulin enter high molecular mass complexes and are released from these complexes capable of forming tubulin heterodimers (Figs. 1–3). The apparent molecular masses of the complexes (≈900 kDa), their lifetimes (≤10 min), and their coelution with TCP1 on both the anion-exchange and size-exclusion columns (cf. Fig. 3 *Inset*) strongly argue that these complexes correspond to the α- and β-tubulin–TCP1 complexes previously detected *in vitro* in rabbit reticulocyte lysate (14). This conclusion was strengthened by immunoprecipitations of the α- and β-tubulin–TCP1 complexes with an anti-TCP1 antibody (Fig. 4). Immunoprecipitations with an anti-β-tubulin monoclonal antibody showed significantly less β-tubulin in fraction I than in fraction III (Fig. 1*C*), despite the fact that two-dimensional SDS/PAGE revealed that fraction I contained the major amount of β-tubulin at early times (Fig. 2; unpublished data). This result suggests a partially buried or altered epitope for β-tubulin when bound to the TCP1 complex, consistent with previous studies in rabbit reticulocyte lysate, which showed that β-tubulin on the TCP1 complex is conformationally different from the free subunit and β subunit in the tubulin heterodimer (14). Thus, it appears that the TCP1 complex functions *in vivo* as a cytosolic chaperonin in the folding of tubulin. Unlike tubulin, native actin does not form dimers. Consequently, we were unable to demonstrate by our chromatographic approach that the actin monomers released from the TCP1 complex in CHO cells (Figs. 2 and 3) are indeed correctly folded. Nevertheless, we believe that the study of Gao *et al.* (12) of actin folding *in vitro*, taken together with our study of actin biogenesis in CHO cells, is sufficiently compelling to conclude that TCP1 is required for actin folding *in vivo*.

Tubulin Dimer Formation *in Vivo*. Free tubulin subunits are the major products of the chase in reticulocyte lysate where endogenous tubulin levels are low (14, 24). Under these conditions, association between free subunits is inefficient and the released subunits accumulate as free α- and β-tubulin (21). In contrast, experiments in CHO cells revealed the tubulin dimer as the major product of the chase (Fig. 2). Since tubulin is a major cytosolic protein (≈4% of soluble protein) (25), tubulin subunits released from the TCP1 complex in CHO cells presumably associate readily with free subunits in equilibrium with endogenous tubulin to form a heterodimer. This model is supported by the observation that when exogenous tubulin is supplied to reticulocyte lysate to increase the free tubulin pool, the major product observed is the heterodimer (14, 21).

It is possible, however, that dimer formation *in vivo* occurs by a different mechanism than in reticulocyte lysate—i.e., via a direct association of α- and β-tubulin subunits on the TCP1 complex. This mechanism could involve preexisting subunits (derived from the equilibrium pool of tubulin) that diffuse to newly folded subunits bound to the TCP1 complex or perhaps an association of newly synthesized α- and β-subunits on the same TCP1 complex. Further studies will be required to distinguish between these models.

Release of Newly Synthesized Actin from the TCP1 Complex. The half-life of the actin–TCP1 complex was estimated from the Superose 6 study to be ≤2–3 min (Fig. 3), whereas the

anion-exchange study showed little change in the intensity of actin in fraction I during a pulse–chase (Fig. 2). This latter result reflects the fact that actin monomer and actin–TCP1 complex elute in almost identical positions from the Mono Q column (data not shown). Interestingly, the ≤2- to 3-min half-life estimated for the actin–TCP1 complex *in vivo* (Fig. 3) is less than the 5- to 10-min half-life reported for this complex *in vitro* (12) and the 5- to 10-min half-life estimated for tubulin–TCP1 complexes both *in vitro* and *in vivo* (Figs. 2 and 3) (14). The basis for these differences is not known.

Substrates of the TCP1 Complex. Actin and tubulin are major substrates for the TCP1 complex in CHO cells (Figs. 3*B* and 4). This observation lends support to the proposal of Gao *et al.* (12) and Yaffe *et al.* (14) that this complex functions as a cytosolic chaperonin for eukaryotes in the folding of actin and tubulin. With the exception of an ≈40-kDa polypeptide (identity presently unknown), we failed to uncover additional proteins that were unambiguously substrates of the complex in CHO cells. Are there sufficient amounts of the TCP1 complex in cells for it to be a general chaperonin? We estimate that there are 150–300 tubulin dimers per TCP1 complex in CHO cells and probably a similar ratio applies for actin (unpublished data). The tubulin dimer and actin are stable proteins with ≈48- and 65-hr half-lives, respectively, in the cytosol (16, 26). If we assume a 6- to 7-min half-life for the α- and β-tubulin–TCP1 complexes and a 2-min half-life for the actin–TCP1 complex in CHO cells, there appears to be sufficient TCP1 complex in the cell to fold tubulin and actin, leaving a small fraction of the TCP1 complex to fold other proteins. We expect ongoing work in this and other laboratories will further elucidate the specificity of the cytosolic chaperonin for substrate and clarify its mechanism of action.

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